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Live-Cell SERS Endoscopy Using Plasmonic Nanowire Waveguides

*Gang Lu, Herlinde De Keersmaecker, Liang Su, Bart Kenens, Susana Rocha, Eduard Fron, Chang Chen, Pol Van Dorpe, Hideaki Mizuno, Johan Hofkens, James A. Hutchison, and Hiroshi Uji-i**

To obtain a better understanding of single, live-cell behavior and cellular communication, it is important to be able to physically or optically address different areas of the cell with high spatial resolution.[1] Optical microscopy, especially fluorescence microscopy, has been intensively employed to investigate cellular processes such as differentiation, propagation and stress response,[1a] and with the advent of optical nanoscopy can now resolve structures with dimensions below the diffraction limit.[1c,2]

An alternative approach is cell endoscopy, in which probes such as glass pipettes, glass fibres, nanowires, or nanotubes are inserted into a cell, allowing not only optical studies but also intracellular payload delivery, electrochemistry, and electrophysiology.[1d,f-h,3] The fabrication of appropriate endoscopic probes is a key challenge here. Optical waveguide probes are increasingly popular as they allow optical information to be easily directed to, and extracted from, the cell.[1g,h,3a,4] For example, fluorescence sensing experiments on living biological specimens have been developed based on optical fibres,[3a,4] but the large, conical probes damage the cell membrane. Recently, endoscopic probes based on SnO_2 nanowires[1g] and photonic nanocavities[1h] have proved to be promising non-invasive probes for optical investigations inside live cells due to their cylindrical form and deformation resistance, however their diameter is still diffraction-limited (200–300 nm for visible light).

Dr. G. Lu, H. De Keersmaecker, L. Su, B. Kenens, S. Rocha, Dr. E. Fron, Prof. H. Mizuno, Prof. J. Hofkens, Prof. H. Uji-i
KU Leuven
Departement Chemie
Celestijnenlaan 200G-F
B-3001, Heverlee, Belgium
E-mail: hiroshi.ujii@chem.kuleuven.be
Dr. C. Chen, Prof. P. Van Dorpe
IMEC, Kapeldreef 75
B-3001, Heverlee, Belgium
Dr. C. Chen, Prof. P. Van Dorpe
KU Leuven
Departement Natuurkunde &Sterrenkunde
B-3001, Heverlee, Belgium
Prof. J. A. Hutchison
ISIS, Université de Strasbourg and CNRS
UMR 7006, Strasbourg, France
Prof. H. Uji-i
PRESTO, Japan Science and Technology Agency (JST)
4–1–8 Honcho Kawaguchi, Saitama 332–0012, Japan

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Metallic waveguides offer a means to go below this size limit as they transfer optical signals via surface plasmon polaritons (SPPs).[1i] SPPs result from the coupling of an optical field with collective oscillations of free electrons at the metal surface and can propagate along the wire before being scattered back to free photons at imperfections or at the wire end. For low-loss metals like silver, single-crystal nanowires of diameter down to 20 nm can be efficient plasmonic waveguides over many microns distance.[5] Moreover if a silver nanoparticle is adsorbed on the silver nanowire (AgNW), SPPs propagating along the wire can efficiently excite surface-enhanced Raman spectroscopy (SERS) from molecules at the junction (or hotspot) between the particle and the wire.[5a] By separating the point of focused laser excitation from the point of SERS detection, this remote excitation methodology (RE-SERS) affords low background spectra with greatly reduced sample photodegradation.[6] Thus both their physical dimensions, and the ability to remotely excite SERS or other photoinduced processes with them, makes AgNWs attractive candidates for probes in live-cell endoscopy, where mechanical stress and phototoxicity are critical issues.

Here we develop AgNW plasmonic waveguide probes for SERS endoscopy on single, live HeLa cells, comparing direct and remote excitation geometries shown schematically in **Figure 1**. Calcium ion stress tests prove the nanowire probes do not mechanically damage the cell membrane during endoscopy, unlike the conical probes (e.g., metallized fibres or AFM cantilevers) typically used in tip-enhanced Raman scattering (TERS) studies.[6] For the remote excitation method (Figure 1b), the sub-diffraction limit excitation volume in the hotspot between the wire and an adsorbed nanoparticle dramatically reduces SERS spectral background and cell phototoxicity compared to direct laser excitation into the cell. In this way, these metallic waveguide probes improve upon current state-of-the-art SERS endoscopes, consisting of capillaries decorated with aggregates of many metallic nanoparticles,[7] and suggest their broader importance for applications in live-cell endoscopy.

AgNWs of ca. 50 nm diameter were synthesized using the polyol method[8] (Supporting Information, Figure S1). The AgNWs were attached to an electrochemically etched tungsten tip (Supporting Information, Figure S2) using the alternating current (AC) dielectrophoresis method,[9] as shown schematically in **Figure 2**a and discussed in further detail in the Experimental section. Several probes can be prepared simultaneously by simply gluing many tungsten wires on an ITO glass electrode using silver paste (Figure 2b), increasing throughput. Compared to other methods such as e-beam welding,[10] our fabrication process is simple and cost-efficient. SEM images of

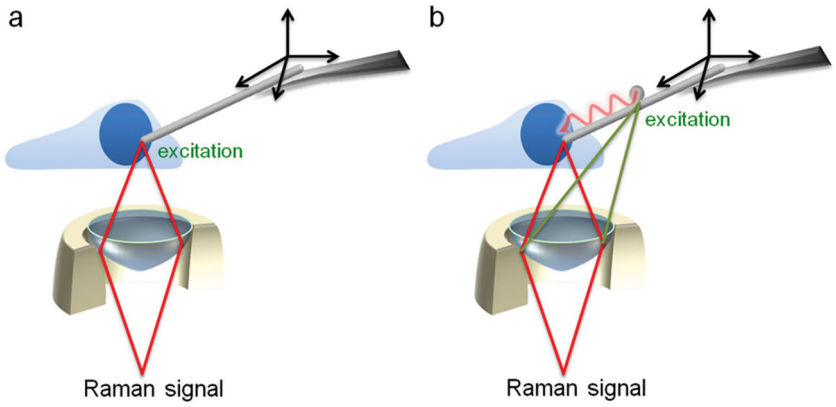

Figure 1. Schematic illustrations of plasmonic waveguide SERS endoscopy using a silver nanowire (AgNW) probe in direct and remote excitation modes. a) In direct excitation mode, a hotspot at the end of the wire, and inside the HeLa cell, is illuminated by a focused 632.8 nm laser beam and Raman signals are collected from the same position. b) In remote excitation mode, the AgNW is illuminated at a hotspot outside the cell, launching surface plasmon polaritons (SPPs) which propagate down the AgNW and inside the cell. Raman signals are detected at hotspots inside the cell that are excited by the propagating SPP.

typical AgNW probes (Figure 2c,d) show that AgNWs were successfully attached in bundles onto the electrochemically etched tungsten tip. Normally however there is only one AgNW at the end of a bundle of AgNWs attached to the tungsten tip. Importantly, within the same batch and among different batches, close to 100% of the tungsten tips have AgNWs attached, indicating our method to be high-yield and highly reproducible. To achieve such high-yield, the AC electrophoresis has to be carried out in a water-ethanol (1:1) solution of AgNWs. Importantly for this work, there are always a few silver nanoparticles (AgNPs) randomly attached to the AgNWs during probe preparation, which can be used for in-coupling light to SPPs, and as hotspot regions for SERS.[5b,6]

In the next fabrication step, the AgNW probes were annealed at 250 °C for 15 min under ambient conditions. After annealing, the probes are quite robust and the AgNWs remain adhered to the tungsten tips even during the application of large deformation stresses (see Supporting Information, Movie S1). Finally, in order to make the probes resistant to immersion in buffer solutions, the junction between the AgNWs and the tungsten tip was glued with epoxy using a micromanipulator equipped with an epoxy-coated tungsten tip (see Supporting Information, Movie S2). The epoxy-glued AgNW probes could then be used for multiple endoscopic experiments.

To carry out live-cell endoscopic measurements, HeLa cells were grown on glass bottom dishes. Before measurements, the growth medium was replaced by Hank's balanced salt solution (HBSS) and the sample placed on an inverted optical microscope (see Experimental section for details). The AgNW probe was slowly inserted into a living HeLa cell with a horizontal angle of about 30° by using a motorized four axis micromanipulator, as shown schematically in Figure 1 and Movie S3 in the Supporting Information.

Since calcium ions (Ca^{2+}) are released into the cytoplasm when the cell membrane is under stress,[11] we measured the stress induced by insertion and release of a AgNW probe by monitoring changes in the concentration of cytoplasmic Ca^{2+} (see Experimental Section for further details). Cells were stained with the fluorescent indicator Calcium Green-1, which emits strong fluorescence upon binding of Ca^{2+}. For comparison, the same membrane stress test was carried out during endoscopy with an electrochemically etched conical tungsten probe (Figure 2e). In the latter case, a dramatically increased (roughly 20 times) fluorescence signal was observed during insertion (Figure 2g) and release (Figure 2h) indicating that the

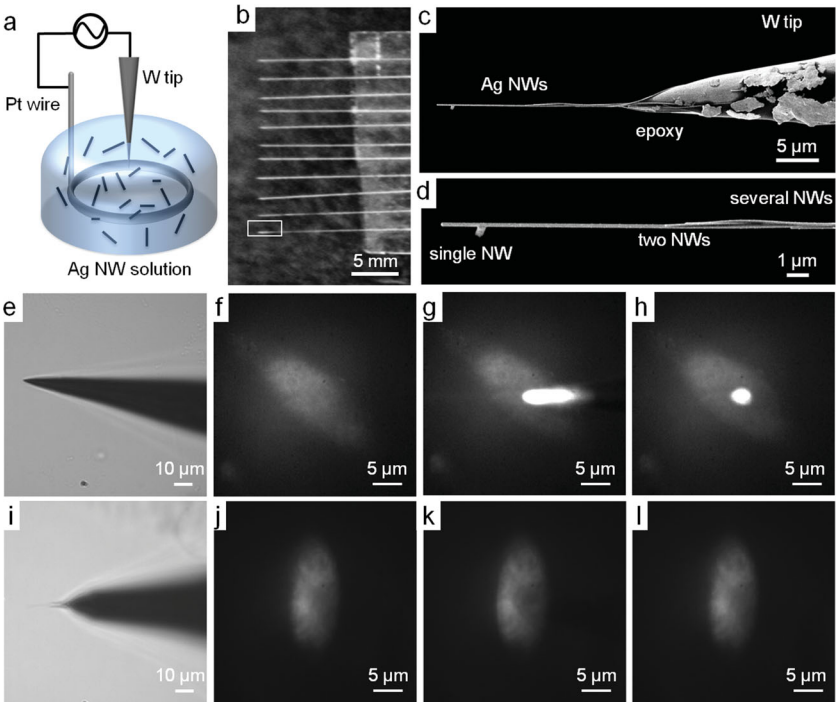

Figure 2. a–e), Fabrication of the AgNW endoscopic probes. a) Scheme of the attachment of AgNWs onto an electrochemically etched tungsten tip using the AC dielectrophoresis method. b) Photo of the AgNW probes obtained, many tungsten wires were glued on ITO glass for high throughput. c) SEM image of a typical tungsten tip with AgNWs attached and glued with conductive epoxy. d) Magnified image of the AgNW probe tip showing a single nanowire at the end of a nanowire bundle (zoom-in area indicated by the white borderlines in (b) and (c). e–l) Calcium ion response of a live HeLa cell during probe insertion and release. Optical transmission image of an electrochemically etched tungsten probe (e); fluorescence images of a HeLa cell before the tungsten probe insertion (f), during probe insertion (g), and during probe release (h). Optical transmission image of an AgNW probe (i). Fluorescence images of a HeLa cell before the AgNW probe insertion (j), during probe insertion (k), and during probe release (l).

cell membrane was significantly damaged by the endoscopy. In contrast, no obvious change in the fluorescence intensity of the Ca^{2+} sensor was observed during insertion (Figure 2k) or release (Figure 2l) of a 50 nm diameter AgNW probe (Figure 2i). Interestingly, endoscopy with 150 nm diameter AgNW probes did not result in obvious fluorescence increase either.

After successful insertion of the AgNW probe into a live HeLa cell, two illumination configurations were employed for collection of Raman signals from inside the cell, the direct and remote excitation modes (Figure 1a and 1b respectively).

In direct excitation mode, a 632.8 nm laser was focused at the free end of the AgNW probe, or at a nanoparticle adsorbed on the AgNW that was inside the cell, and SERS signals were collected from the same position (Figure 1a). Multiple nanoparticle hotspots on the AgNW probe could be illuminated with the laser beam, such that Raman signals could be collected from different areas outside and inside the HeLa cell (**Figure 3**a). As shown in Figure 3b, no obvious SERS signals were observed from the HBSS buffer solution, which is reasonable since the Raman cross section of the salts present in the HBSS buffer

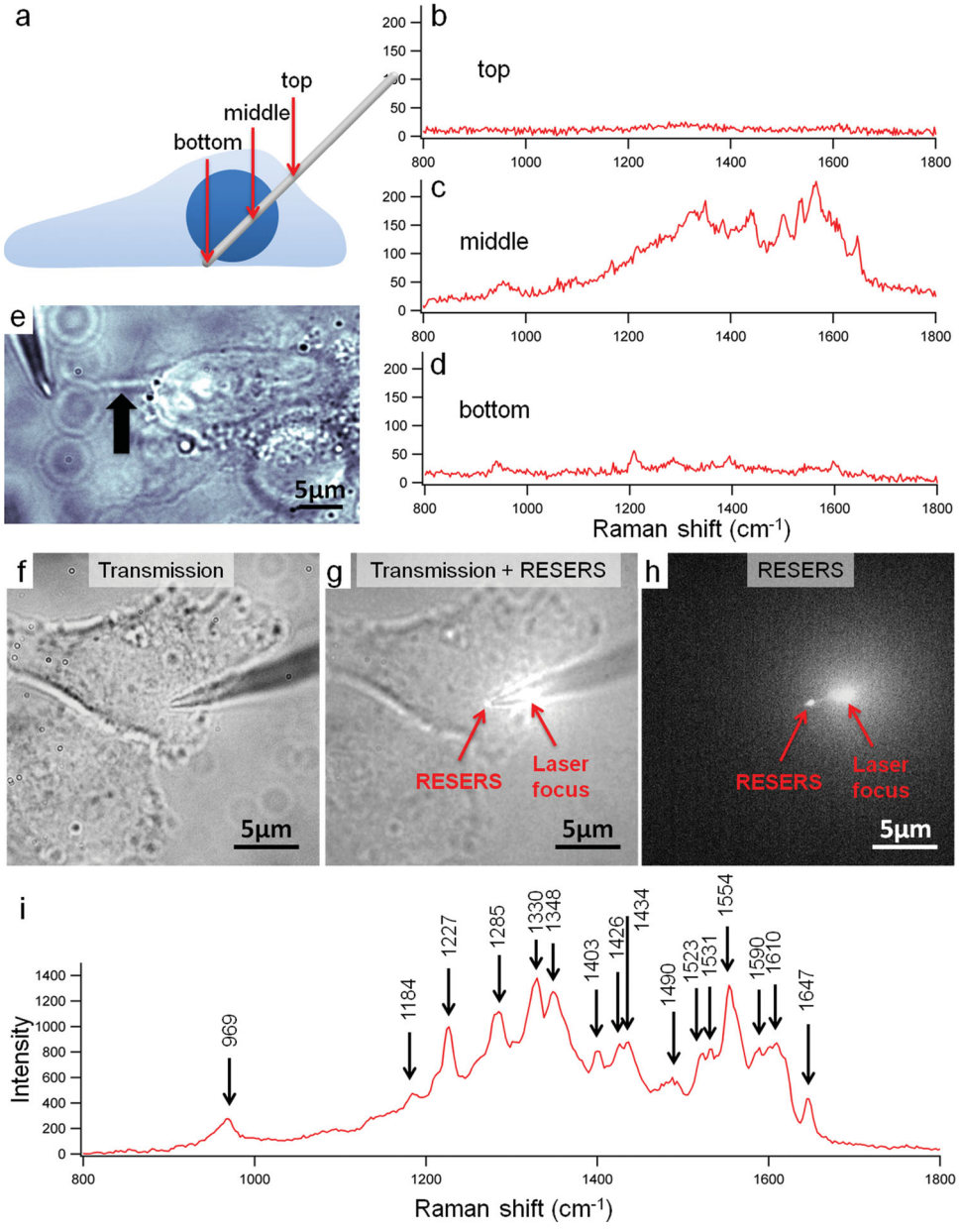

Figure 3. a–e), Direct excitation SERS endoscopy of a live HeLa cell. a) Scheme of the positions chosen for direct excitation SERS spectroscopy inside a live HeLa cell during AgNW probe endoscopy. b–d) Raman spectra taken from hotspots at the top (b), middle (c) and bottom (d) parts of the AgNW probe corresponding to regions in the HBSS buffer, the cell nucleus, and the cell cytoplasm, respectively. e) After direct excitation SERS endoscopy measurement, cell components adhere to the AgNW probe and come away with the probe during release (indicated by the arrow). f–i), Remote excitation SERS endoscopy of a live HeLa cell. Optical transmission (f), combination of optical transmission and remote excitation SERS (g) and remote excitation SERS only (h) images of a AgNW probe in a live HeLa cell. i) A remote excitation SERS spectrum from the nucleus of the live HeLa cell.

is very low. In contrast, several clear Raman peaks, on top of a large background, were observed from a hotspot inside the cell nucleus (Figure 3c).These fingerprint region Raman peaks, from 900 to 1700 cm^{-1}, may come from the protein and/or DNA inside the cell nucleus,[12] while the background is likely due to Raman signal from carbon, enhanced fluorescence or image-molecule/electronic continuum coupling frequently seen in SERS spectra.[13] At a hotspot in the cell cytosol, only a few weak SERS peaks were observed (Figure 3d). This is expected since the cytosol is a complex mixture of substances dissolved in water which mostly have small Raman cross sections.

These results demonstrate that the AgNW probe can detect distinct SERS signals from different regions inside a single living cell. However even in the cell nucleus, where strong SERS signals are measured, direct excitation results in a huge background in the SERS spectrum, and certainly excessive heating and light scattering at the illumination spot, which may affect normal cellular function. Indeed, during probe extraction after direct excitation SERS endoscopy, cell components adhere to the AgNW and come away together with it (Figure 3e). After this event, cells often become round and detach from the substrate indicating cell death.

To overcome these problems, we also conducted SERS endoscopy using the remote excitation configuration (Figure 1b). SERS hotspots on the AgNW probe inside the cell were excited by propagating SPPs, launched from a part of the AgNW probe outside the cell (by focusing the laser either at the end of the AgNW, at a AgNW-AgNW junction, or at nanoparticles adsorbed on the AgNW). Remote excitation SERS from a hotspot in the HBSS buffer again showed no clear Raman peaks (Supporting Information, Figure S3). However, when an AgNW probe hotspot was inserted into a live HeLa cell nucleus (Figure 3f–i), a distinct remote excitation SERS spectrum was observed (Figure 3i). In the transmission images, the NW appears conical, because the shadow of the tilted NW, which was tilted roughly 60° from the optical axis, was projected on the image plane. In contrast to the direct excitation SERS spectrum (Figure 3c), the peaks in the fingerprint region from 900 to 1700 cm^{-1} can be clearly resolved and thus could be used to identify DNA and/or proteins inside the nucleus.[12,14] Critically, in remote excitation mode, the cell contents only rarely stick to the AgNW probe during probe release and the cell survives the endoscopy, making studies over time possible.

In summary, plasmonic waveguides based on silver nanowires were successfully employed as endoscopic probes for SERS spectroscopy of live HeLa cells. A cost-efficient AC dielectrophoresis method was used for the preparation of AgNW probes with high yield and high reproducibility. These probes, with diameters of 50 and even 150 nm, were shown to cause significantly less stress to live cells during endoscopy than conventional conical probes. SERS signals at hotspots on the AgNW probes were collected from different regions inside the HeLa cells, with the cell nucleus giving the strongest signals, however only the remote excitation technique afforded clearly resolved spectra and reduced cell photothermal/chemical damage by shifting the diffraction limited laser focus away from the cell.

Many avenues remain open for improving the remote excitation SERS endoscopy methodology. Engineering the SERS hotspots on the AgNW probe can increase the sensitivity of intracellular chemical identification. This may be achieved by careful choice of adsorbing nanoparticles (size/metal), or by directly texturing the nanowire itself. In either case, the hotspot must be accessible for sensing the contents of the cell, but confined enough to ensure large SERS enhancement. Conversely in areas such as the nucleus where strong SERS signals due to multiple species are detected, increasing the specificity of a SERS hotspot for a target bio-marker by binding a receptor is an important priority of future research. Nevertheless, the use of sub-diffraction limit diameter plasmonic waveguides as endoscopic probes clearly has enormous potential application in the study of live-cell behavior. Looking beyond SERS spectroscopy, utilizing the nanowire probes as point sources for luminescence spectroscopies, or for initiating photochemical reactions (beneficial or deleterious) at highly spatially resolved positions inside a live cell, are other very promising possibilities.

Experimental Section

Detailed Preparation of the AgNW Probes: First, a tungsten tip was prepared by applying an AC voltage, with frequency 50 Hz and amplitude 3 V, between a tungsten wire of diameter 50 μm (Advent Research Materials) and a ring-shaped platinum wire electrode of diameter 8 mm (Advent Research Materials) in a 2 M aqueous solution of sodium hydroxide (Sigma–Aldrich). A typical SEM image of the prepared tungsten tip is shown in Figure S2. After rinsing with Milli-Q water, the etched tungsten tip was immersed in an aqueous solution of AgNWs (50 nm diameter, see Figure S1) that were synthesized using the polyol method,[8a,8b] washed with ethanol 3 times, and diluted with Milli-Q water 100 times. Then an AC voltage with frequency of 1 MHz and amplitude of 8 V was applied between the tungsten tip and the ring-shaped platinum wire electrode for ca. 10 s to obtain the AgNW-attached tungsten tip (Figure 2a–d). A few Ag nanoparticles were also randomly attached onto the AgNWs during the process. In order to bind the AgNWs strongly to the tungsten tip, the probe was annealed at 250 °C for 15 min on a hotplate, then the junction between the AgNWs and the tungsten was glued with conductive epoxy using a motorized four-axis micromanipulator (MX7600, GMP) equipped with an epoxy pre-coated tungsten tip (see Supporting Information, Movie S2). The prepared AgNWs-attached tungsten tip (the AgNW probe) was then used in live-cell endoscopy experiments.

Live-Cell Endoscopy Experiments: HeLa cells[15] were maintained in Dulbecco's modified eagle medium (DMEM) (from Invitrogen) supplemented with 10% fetal bovine serum (FBS), GlutaMAX supplement and 50 μg mL^{-1} gentamicin at 37 °C and 5% CO_2 environment. For imaging, the cells were grown on a 35 mm glass bottom dish (P35G-0–14-C, MatTek). Before imaging, the cell sample was washed three times with 1 mL of HBSS and the glass bottom dish was then placed on an inverted microscope (TiU, Nikon). Afterward, an AgNW probe was slowly inserted into the live HeLa cell, with an angle of about 30°, controlled by a motorized four-axis micromanipulator (MX7600, GMP, see Supporting Information, Movie S3). The diffraction-limited focused light excitation was provided by a continuous wave 632.8 nm He–Ne laser (1145P, JDSU). Circular polarization at the sample was achieved by tuning half-wavelength ($\lambda/2$) and quarter-wavelength ($\lambda/4$) wave plates. The Raman signals were collected by a spectrometer (iHR 320, Horiba) equipped with a cooled electron multiplying charge-coupled device (CCD) camera (Newton 920, Andor). Typically, the laser power used was ca. 1–10 kW cm^{-2}, and the image integration time was 5 s.

Ca^{2+} Response Studies: For cell stress tests, the cell sample was stained with Calcium Green-1 AM (Invitrogen). Briefly, a solution containing the calcium indicator (5 μM) and Cremophor EL (0.01% w/v) was added to above-mentioned cell sample in HBSS. Cremophor EL was added

to facilitate the uptake of the dye. The cells were incubated at room temperature for 30 min and washed 3 times with HBSS. Fluorescence images of this stained cell sample during probe insertion and release were observed using an inverted microscope (TiU, Nikon) equipped with a CCD camera (C9100–13, Hamamatsu Photonics). Wide-field illumination was provided by a 488 nm diode-pumped solid-state laser (Sapphire 488 SF-100CW, Coherent).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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